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DNA fiber assays

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An abbreviated version of this protocol was published in Science Advances in Jun 2020

Inhibition of the translesion synthesis polymerase REV1 exploits replication gaps as a cancer vulnerability

DOI: 10.1126/sciadv.aaz7808

Detailed protocol

DNA Fiber Assay to determine replication fork speed

Day 1

1. Seed 1 x 10^6 cells per 10cm plate for each treatment condition.

Day 2

1. Remove the media from the plate and treat the cells with either DMSO or drug of interest (300nM KSQ-4279/ 300nM Olaparib) for either 6hr or 24hr or 72hr.
2. Upon treatment, remove the media and wash the cells once with sterile 1X PBS.
3. To the plate, now add fresh media with the same drug of interest + 50uM IdU. Keep the plate at 37C incubator for 30mins. This is the IdU labeling step in presence of the drug.
4. Remove the media with IdU and gently wash the cells three times with sterile 1X PBS.
5. Now add fresh media with the same drug of interest + 150uM CldU(150uM) and incubate the plate at 37C for 30mins. This is the CldU labeling step in presence of the drug.
6. Remove the media with CldU and gently wash the cells three times with sterile 1X PBS.
7. Trypsinize the cells with 0.25% Trypsin-EDTA for 2-5mins (The time depends on when you see all the cells getting detached from the plate and free floating).
8. Collect the cell suspension and centrifuge at 4C for 5mins at 1000 rpm.
9. Remove the supernatant and wash the cell pellet with 1X PBS. Spin it again at 4C for 5mins at 1000rpm.
10. Carefully remove the supernatant and resuspend the cell pellet in 4ml of 1X PBS.
11. Mount a drop (2.5ul) from the cell suspension on positively charged microscopic slide. You can mount 2-3 drops per slide. Make sure to add the drop closer to the frosted area. This allows the drop to spread and create DNA fibers.
12. To those drops add 7.5ul of lysis buffer (200mM Tris-HCL pH 7.5, 50mM EDTA, 0.5% SDS) and gently pipette up and down 4-5 times to mix and lyse the cells. Incubate the drop for exactly 7-8mins at RT and not more.
13. Tilt the slide at 45degree angle to allow the drop that now contains the DNA from the cells to run down the slide.
14. Wait and let the drop to air dry for 15-20mins which will result in a white smudge or line across the slide.
15. Fix the DNA by keeping the slide in a coplin jar with freshly prepared and ice-cold Methanol:Acetic Acid (3:1) for 5mins.
16. Let the slides dry and store at 4C or proceed with the staining.

Day 3: Staining the DNA fibers (Remember to perform these steps in dark and avoid exposure to light)

1. Wash the slides twice with 1X PBS for 5mins each in a coplin jar.
2. Denature the DNA for 1hr by treating with 2.5M HCL at RT in a coplin jar.
3. Wash the slides three times with 1X PBS for 5mins each to neutralize the acid in a coplin jar.
4. Treat the slides with blocking buffer (3%BSA made in 1X PBS + 0.1% Triton-X) for 30-45mins at RT in a coplin jar.
5. Wash twice with 1X PBS and proceed with primary antibody staining in a coplin jar.
6. Incubate with 50ul of primary antibodies for 2.5hrs at RT in a humid chamber.

[Remember to make the antibody dilutions in the blocking buffer. Make sure that the antibody covers the entire surface of the slide. This can be achieved by gently placing a parafilm cut in the size of the slide over as a coverslip to avoid drying.]

Primary Antibodies used:

1. IdU [Becton Dickinson 347580 – Mouse monoclonal anti BrdU 1:100]
2. CldU [Abcam ab6328 – Rat anti BrdU 1:100]

7. Put 500ul of 1X PBS on the slides to gently remove the parafilm.

8. Wash the slides with 1X PBS for 5mins for a total of three times.
9. Incubate with 50ul of secondary antibodies for 1hr at RT in a humid chamber. Make sure that the antibody covers the entire surface of the slide by gently placing a parafilm cut in the size of the slide over as a coverslip to avoid drying

Secondary Antibodies used:
IdU [Alexa 488 – Goat anti mouse 1:200]
CldU [Alexa 594 – Goat anti rat 1:200]

10. Repeat steps 7 and 8.
11. Dry the slides and add 20ul of Prolong gold Antifade (Invitrogen, P36930) and add a coverslip.
12. Let the slide dry at RT in the dark and then store at 4C (at -20C for longer storage).
13. Image the slide under 60X oil [Channels used FITC and TRITC] [Deltavision scope at Microscope facilty, W.M.Keck Institute].

DNA Fiber Assay to determine replication fork degradation

Day 1
1. Seed 1 x 10^6 cells per 10cm plate for each treatment condition.

Day 2
1. Remove the media from the plate and treat the cells with IdU (50uM) (diluted in media). Keep the plate at 37C incubator for 30mins.
2. Remove the media with IdU and gently wash the cells three times with sterile 1X PBS.
3. Add media with CldU(150uM) to the plate and incubate at 37C for 30mins.
4. Remove the media with CldU and gently wash the cells three times with sterile 1X PBS three.
5. Add media with DMSO or drug of interest (300nM KSQ-4279/ 300nM Olaparib/ 0.5mM Hydroxyurea either alone or in combination) to the plate and incubate at 37C for 6hrs.
6. Carefully remove the media with DMSO or drug of interest and gently wash with sterile 1X PBS three times.
7. Trypsinize the cells with 0.25% Trypsin-EDTA for 2-5mins (depending on when you see the cells detached from the plate and are free floating).
8. Collect the cell suspension and centrifuge at 4C for 5mins at 1000 rpm.
9. Remove the supernatant and wash the cell pellet with 1X PBS. Spin it again at 4C for 5mins at 1000rpm.
10. Carefully remove the supernatant and resuspend the cell pellet in 4ml of 1X PBS.
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16. Let the slides dry and store at 4C or proceed with the staining.

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